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(54) Title: A PROCESS FOR PREPARING PEPTIDE NUCLEIC ACID PROBE USING POLYMERIC PHOTOACID GENERATOR

(57) Abstract: The present invention relates to a process for preparing arrays of oligopeptide nucleic acid probes immobilized on a solid matrix by employing polymeric photoacid generator. Arrays of peptide nucleic acid probes of the invention are prepared by the steps of: (i) derivatizing the surface of a solid matrix with aminoalkyloxysilane in alcohol and attaching a linker with acid-labile protecting group on the solid matrix; (ii) coating the solid matrix with polymeric photoacid generator (PAG); (iii) exposing the solid matrix thus coated to light to generate acid for eliminating acid-labile protecting group; (iv) washing the solid matrix with alkaline solution or organic solvent and removing residual polymeric photoacid generator; and, (v) attaching a monomeric peptide nucleic acid with acid-labile protecting group to the solid matrix, and repeating the previous Steps of (ii) to (v). In accordance with the present invention, neutral peptide nucleic acid probes, as the promising substitute for conventional negatively-charged oligonucleotide probes, can be prepared by employing polymeric photoacid generator in a simple and efficient manner, while overcoming the problems confronted in the prior art DNA chip fabrication using PR system and PPA system.

A PROCESS FOR PREPARING PEPTIDE NUCLEIC ACID PROBE USING POLYMERIC PHOTOACID GENERATOR

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to a process for preparing peptide nucleic acid probes by employing polymeric photoacid generator, more specifically to a process for preparing arrays of oligopeptide nucleic acid probes immobilized on a solid matrix by employing polymeric photoacid generator.

Description of the Prior Art

Biochip, a kind of biosensor used for genomic analysis, medical diagnosis and monitoring biological processes and environment, is largely classified into DNA chip which identifies nucleotide sequences and protein chip which recognizes proteins such as pathogens, antibodies, antigens or enzymes.

The structure of DNA identified by Watson and Crick in 1953 has been a great impact on life sciences such as molecular biology, biochemistry, etc. DNA is a biopolymer with four different bases of adenine(A), cytosine(C), guanine(G), thymine(T), sugar(deoxyribose) and phosphate, to build a very stable double helix structure: the phosphate-sugar forms backbone, bases attached to the sugar are paired with complementary bases, such as A to T, and G to C, which are stabilized by hydrogen bond. The specific/complementary hydrogen bond between bases plays a very important role in drug developments such as antisense drug and gene therapy, in particular, for genetic disease, cancer and cardiac diseases.

Recently, in line with the great efforts made to

identify approximately 100,000 human genes, needs for the
methods for providing enormous genomic information as fast
as possible for the diagnosis and prevention of genetic
disease are increasing. Despite Sanger's DNA sequencing
method is improved by polymerase chain reaction and
automation, it still remains cumbersome and time-consuming,
and requires high cost as well as highly trained personnel.
Therefore, alternative method to overcome those shortfalls
has been constantly deliberated in the art. By virtue of
such needs, there has been a considerable progress in the
fabrication and utilization techniques of DNA chips for
recent several years.

In general, DNA chip is high-density micro-arrays of
known oligonucleotide probes ranging from several hundred
to several hundred thousands immobilized on a smaller than
one square inch solid surface, such as silica, surface-
derivatized glass, polypropylene or activated
polyacrylamide. If the target DNA fragments are placed on
DNA chip, the target DNA fragments hybridize to the probes
on the chip according to the sequence complementarity.
Therefore, the sequence of the target DNA can be analyzed
by the presence and location of hybridized DNA detected by
the optical or radiochemical method. Using the DNA chip
aided method, DNA analysis system can be miniaturized such
that an extremely small amount of sample DNA can be used
for diagnosis, several sequences in a target DNA can be
analyzed simultaneously, and thus the genetic information
can be obtained in a simple, cost effective and fast manner.

The sequence analysis technique using
oligonucleotide DNA chip is an innovative method since it
is faster and easier to use for sequencing a target gene
than the conventional Sanger's method. The Sanger's
sequencing method, which requires the separation of
fluorescence- or radioisotope-labeled DNA fragments by gel
electrophoresis, is proven to be less satisfactory in light
of time-consuming and technical difficulties. On the
contrary, sequencing by hybridization(SBH) using

oligonucleotide DNA chip employs a principle that by placing fluorescence- or radioisotope-labeled target gene fragments to a DNA chip with oligonucleotide probes, and then simply by washing the chip with solvent, gene fragments complementary to known oligonucleotide probes are attached by hydrogen bond.

Merryfield's synthetic method in which chemical compounds are reacted on organic solvent-resistant solid matrix and further organic reactions are carried out on the solid matrix, has been used effectively in synthesizing oligopeptide of biologically important enzymes and oligonucleotide of genes, since it is very simple and efficient in the purification of reaction products(see: Fed. Proc., 24:412, 1962). A new efficient synthetic method combining the said method with combination chemistry has been used effectively for catalyst screening and also been used in drug development as well. In addition, by the combination of the synthetic method on a solid matrix and the photolithography employed in semiconductor industry, various techniques for preparing arrays of oligonucleotide probes have been developed to be used for genetic diagnosis. The said technique comprises selective activation of specific regions of surface-derivatized glass to which the oligonucleotide binds chemically. To activate the specific targeted region, the light beam is applied through a transparent region of photolithographic mask made by a predetermined pattern. By controlling the mask pattern and composition of nucleic acids at each step, a specific nucleic acid can be positioned on a desired location. This technique is an ultra-fine processing technique used in semiconductor devices which makes possible several millions of probes be affixed on a fingernail-size chip.

For example, Fordor et al teaches a new direct photolysis technique, where nucleic acid or amino acid with UV-labile protecting group is attached on a solid surface, the protecting group is eliminated by exposing the selected regions of surface to light using photolithographic mask,

which is subsequently reacted with a new nucleic acid or amino acid with a photolabile protecting group, to polymerize nucleic acid or amino acid at specific location(see: USP 5,445,934). Since this method allows
5   selective systhesis of oligonucleotide probes with a specific sequence/length at a specific location, it is useful in synthesizing various oligonucleotide probes with a desired sequence and length at a predetermined position. Also, since this method employs ultra-fine process used in
10   semiconductor devices, it is extremely useful for fabricating oligonucleotide probes in high density. Fordor et al also suggested that a sequencing method using the oligonucleotide probes which is much easier and faster than Sanger's method, is useful for making high density
15   oligonucleotide probes. However, it has revealed a shortcoming that: the removal of photolabile protecting group is proportional to the power of a light source, which plays a detrimental role in the ultra-fine process for making high density chips.
20   On the other hand, photolithographic process using photoresist(hereinafter referred to as 'PR') which is used for micropattern formation in semiconductor industry has attracted attention as an essential technique to improve the density of DNA chip. Since the size or capacity of a
25   semiconductor chip is depending on the spatial resolution of photolithographic process, photolithographic process has played a leading role in the semiconductor and microelectronics industry. The photolithographic process utilizes the solubility differences of PR between the light
30   exposed region and the unexposed region. The solubility reduction in the exposed region is called as negative system and solubility increase is called as positive system, and the latter is used mostly for the production of semiconductor chips. By using above photolithographic
35   process, more oligonucleotide probes can be arrayed on a limited area of chip.
        Up to now, photolithographic process has been applied

in the general PR system(see: USP 5,658,734) and the photoacid patterned array system(hereinafter referred to as 'PPA', USP 5,843,655) as well.

Photolithographic process using PR system(hereinafter referred to as 'PR process') has an advantage of using materials already developed or commercialized for semiconductor industry. According to the system, a pattern is formed by the light exposure and washed out to lead to standard solid-phase nucleic acid synthetic reaction on the surface, finally to link nucleotides. The PR includes diazoquinone/cresol-novolac, highly adhesive to the surface, which is shown to have good pattern characteristics in i-line(365nm) and used in 16 mega DRAM processing. However, the PR is developed in alkaline solution ([OH-]>0.1M), that causes the cleavage of amide bond protecting the amino group of base. The protecting coating under PR has been suggested to overcome the said problem of development(see: J. Vac. Sci. Tech., B7(6):1734, 1989).

PR process is consisted of three major steps: the first is mainly the PR pattern formation with PR coating, light exposure and developing; the second is the removal of protecting group and unexposed area by acidic solution; and, the third is the attachment of nucleic acid and post-treatment. Photolithographic process using PR system is good enough for obtaining high resolution, but complex processing as described above has been a major drawback.

To simplify the complex process and improve the efficiency, the PPA system has been proposed in the art. Since the PPA system uses polymeric matrix mixed with photoacid generator(hereinafter referred to as 'PAG'), acids are generated only at the exposed region and removal of protecting group occurs after heat treatment, therefore the two separate steps employed in PR processing can be carried out in one step. However, this PPA system has revealed several problems as well. For example, while PR process is not affected by the concentration or volume of the acid solution since it uses large volume of acid

solution to contact and to remove the protecting group, acid generated by the PPA system remains on the polymeric matrix and, to balance the amount of acid, more PAG should be added. However, at above a certain level of PAG, PAG domain forms and scatters light which in turn interferes with micro pattern formation. In other words, excessive amount of PAG which is used for stability and reproducibility of the pattern formation reduces the efficiency of processing. In addition, the generated acid should diffuse from the polymer to the surface(actually closer to the heat source) of the protecting group by heat, but the generated acid, when the glass plate is heated on a hot plate, may diffuse to the opposite direction from the protection group, thereby reducing the efficiency. Furthermore, the mixed film of polymer and PAG for coupling, has to be removed by organic solvent such as acetone or methylethylketone, which makes the progress more expensive.

Another problem confronted in the art DNA chip techniques is the reduction of degrees of the association/dissociation, speed in the course of hybridization of oligonucleotide probes with target genes and point mutations present in a gene. For example, Nielsen et al developed neutrally charged peptide nucleic acid(hereinafter referred to as 'PNA') to replace oligonucleotides which can be synthesized by the modified Marryfield's peptide synthesis method, based on the findings that the repulsive force between negatively-charged oligonucleotides reduces the annealing strength as well as speed during hybridization of oligonucleotides. PNA greatly improved the association power, and speed during hybridization with oligonucleotide and it is practically applied in detecting single base mutation in a particular gene, which can be realized by the reduced association between non-complementary oligonucleotide(see: Nature, 8:53, 1993). Due to this positive characteristic, the antisense drug using PNA is under investigation. However, despite the benefits, there has been no report of

using PNA on high density peptide nucleic acid probe synthesis by photolithographic process.

Therefore, there are strong reasons for developing and exploring alternative method for synthesizing peptide nucleic acid as well as oligonucleotide with various nucleotide sequences on a solid matrix, while overcoming the problems confronted in the prior art DNA chip fabrication using PR and PPA system.

SUMMARY OF THE INVENTION

The present inventors made an effort to provide an efficient method for preparing peptide nucleic acid probes with various nucleotide sequences, and developed a highly efficient process for preparing arrays of peptide nucleic acids, which can eliminate the repulsion problem associated with negatively charged oligonucleotide, immobilized on a solid matrix by employing polymeric photoacid generator(hereinafter referred to as 'polymeric PAG') while overcoming the problems of the prior art PPA process employing PAG in polymer.

A primary object of the present invention is to provide a process for preparing arrays of peptide nucleic acid probes with various nucleotide sequences on a solid matrix by employing polymeric photoacid generator.

The other object of the present invention is to provide arrays of peptide nucleic acid probes with various nucleotide sequences prepared by employing polymeric photoacid generator.

DETAILED DESCRIPTION OF THE INVENTION

The peptide nucleic acid probes with various nucleotide sequences are prepared by derivatizing the surface of a solid matrix with aminoalkyloxysilane in

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alcohol and attaching a linker with acid-labile protecting group on the solid matrix, coating the solid matrix with polymeric photoacid generator(PAG), exposing the solid matrix thus coated to light to generate acid for
5   eliminating acid-labile protecting group, washing the solid matrix with alkaline solution or organic solvent and removing residual polymeric photoacid generator, and, attaching a monomeric peptide nucleic acid with acid-labile protecting group to the solid matrix, and repeating the
10 previous steps.

Polymeric PAG employed in the invention is a polymer with molecular weight of 500 to 1,000,000, which has sulfonium salt on backbone or side chain, and has also organic photoacid generating group on side chain to
15 generate acid by exposing to light.

The peptide nucleic acid monomers of the invention has N-(2-aminoethyl) glycine backbone to which adenine, cytosine, guanine, or thymine base is linked by amide bond. Peptide nucleic acids are synthesized by amide bond between
20 an amino group of backbone and a carboxyl group of other peptide nucleic acid monomer. Currently, peptide nucleic acid monomers protected by acid-labile t-butyloxycarbonyl protecting group or alkali-labile fluoromethyloxycarbonyl protecting group are commercially available, where
25 exocyclic amino groups of adenine, cytosine and guanine are protected by acid-stable diphenylmethyloxycarbonyl or benzyloxycarbonyl protecting group. The present inventors employed monomers whose amino groups of backbone are protected by acid-labile t-butyloxycarbonyl, and
30 synthesized bases whose exocyclic amino groups are protected by stable p-methoxybenzoyl or isobutanoyl group.

The peptide nucleic acid synthesis method is generally carried out in a similar manner as the oligonucleotide synthesis method conventionally known in
35 the art(see: Acc. Chem. Res., 24:278, 1991). Nielson et al synthesized oligopeptide nucleic acid by using solid-phase phase as follows: First, the amino group of solid support

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is reacted with the carboxyl group of peptide nucleic acid of specified base(A, C, G or T) whose amino group in backbone is protected by acid- or base-labile functional group to link each other in a form of amide bond. Next, the resultant is treated with acid or base to eliminate amino protecting group to reveal amino group, which is subsequently reacted with the carboxyl group of peptide nucleic acid of specified base whose amino group in backbone is protected by acid- or base labile functional group to link each other in a form of amide bond, and, the said steps are repeated to obtain an oligopeptide of desired base sequence and number, and finally treated with strong acid to separate the exocyclic amino protecting group from solid support by chemical reaction. This method is desirable in a sense that it assures complete reaction of excessive peptide nucleic acids(5 equivalents) as much as possible and easy purification of peptide nucleic acid on an organic solvent-resistant solid support by filtering the residual monomers and reactants and washing with organic solvent. Based on the solid-phase synthesis, the present inventors prepared biochip with various nucleotide sequences to finally prepare arrays of peptide nucleic acid probes immobilized on a solid matrix by employing polymeric PAG process illustrated above.

The polymeric PAG and peptide nucleic acid monomer which are employed in the process of preparing peptide nucleic acid probes with various nucleotide sequences were prepared by Preparative Examples 1 and 2, and Preparative Example 3, respectively.

Preparative Example 1: Polymeric PAG in chloride salt form

0.026 Mole of anisol diphenol sulfide salt was dissolved in 26ml of N,N'-dimethylacetamide, added 0.077 mol of triethylamine, then cooled to 0°C. To 0.026mole of sebaconylchloride in 26ml of heptane, was added the said

solution, and reacted overnight at room temperature. After the top layer of heptane was separated and gently removed from the mixture, the N,N'-dimethylacetamide layer was added in a dropwise to 400ml of diethylether to obtain the precipitate. The polymeric PAG in chloride salt form was obtained by filtering and drying the precipitate. The material thus obtained was resolubilized in 20ml of methanol, re-precipitated in water, and then filtered and dried to obtain the titled compound.

Preparative Example 2: Substitution of salt

Preparative Example 2-1: p-Toluenesulfonic salt of polymeric PAG

0.01 Mole of polymeric PAG in chloride salt form obtained in Preparative Example 1 was dissolved in 10ml of methanol, added 0.03mol of p-toluenesulfonic acid in sodium salt form. After 3 hours' incubation, the solution was concentrated by removing 7ml of methanol and precipitated by adding water. The precipitate was filtered and dried to obtain p-toluenesulfonic acid salt of polymeric PAG.

Preparative Example 2-2: Naphtol sulfonic acid salt of polymeric PAG

0.01 Mole of polymeric PAG in chloride salt form obtained in Preparative Example 1 was dissolved in 10ml of methanol, then added 0.03mol of 1-naphthol-5-sulfonic acid in sodium salt form. After 3 hours' incubation, the solution was concentrated by removing 7ml of methanol and precipitated by adding water. The precipitate was filtered and dried to obtain 1-naphthol-5-sulfonic acid salt of polymeric PAG.

Preparative Example 2-3: 9,10-Dimethoxy-2-anthracene sulfonic salt of polymeric

PAG

0.01 Mole of polymeric PAG in chloride salt form obtained in Preparative Example 1 was dissolved in 10ml of methanol, then added 0.012mol of 9,10-dimethoxy-2-anthracene sulfonic acid salt. After 3 hours' incubation, the solution was concentrated by removing some methanol and precipitated by adding water. The precipitate was filtered and dried to obtain 9,10-dimethoxy-2-anthracene sulfonic acid salt of polymeric PAG.

Preparative Example 3: Peptide nucleic acid monomer

Preparative Example 3-1: Peptide nucleic acid backbone

In a 2 liter round flask, 107ml(1.60mol) of ethylenediamine and 60ml of 1,4-dioxane were mixed with stirring. Then, 600ml of di-t-butylcarbonate solution (43.65g(0.20mol) in 1,4-dioxane) was added and incubated overnight with constant stirring. Then, the solvent was evaporated under reduced vacuum and added 1 liter of water to the remaining solution. The white precipitate generated by addition of water was removed by filtering and extracted with dichloromethane. Organic phase layer thus obtained was pooled and dried with magnesium sulfate, and concentrated under reduced vacuum to obtain 27.21g(1.36mol) of t-butyl N-(2-aminoethyl) glycinate, white powder. 5.00g(0.25mol) of t-butyl N-(2-aminoethyl) glycinate was dissolved in 500ml of dichloromethane in a 2 liter reaction flask, then added 41.5ml(0.30mol) of triethylamine at once, and added 62.54g(0.27mol) of benzyl-2-bromoacetate in a dropwise, and incubated overnight with constant stirring. The resultant was then extracted with dichloromethane, washed with water and brine in a serial manner. Then, the resulting organic phase layer was dried with magnesium sulfate, and concentrated under reduced vacuum to obtain crude compound. The crude compound was fractionated with

silica gel column chromatography by using a mobile phase of 5% methanol/dichloromethane, and 47.80g(0.16mol) of benzyl N-[2-(t-butoxyl)aminoethyl] glycinate was obtained in a white powder form.

^{1}H NMR(CDCl$_3$) δ 7.39(s, 5H), 5.18(s, 2H)

        5.01(br s, 1H), 3.5(s, 2H)

        3.25(t, 2H), 2.78(t, 2H), 1.45(s, 9H)

Preparative Example 3-2: Adenine monomer

After 27.03g(0.20mol) of adenine was dissolved in 500ml of pyridine, 34.12g(0.20mol) of p-anisole chloride was added slowly over 55 minutes, and reacted for 4 hours at 100°C with stirring. Then, the reaction temperature was lowered to room temperature, further reacted overnight with constant stirring, and removed pyridine by distilling under reduced vacuum. The resulting crude compound was recrystallized in isopropanol and methanol respectively, to give 33.7g(0.13mol) of white powder of N^ϵ-(4-methoxybenzoyl) adenine. 33.66g(125mmol) of N^ϵ-(4-methoxybenzoyl) adenine and 400ml of DMF were placed in a 1 liter reaction flask, added 6.0g(150mmol) of 60% NaH slowly, added 22.15ml(150mmol) of t-butylbromoacetate in a dropwise over 1 hour 15 minutes, and then, reacted overnight. The reaction solvent, DMF was removed under reduced vacuum, and the precipitate was obtained after addition of water. The precipitate was separated and recrystallized in methanol to obtain 33.46g(87.30mmol) of N^ϵ-(4-methoxybenzoyl)-9-(t-butoxycarbonylmethyl) adenine as a white solid. In a 2 liter reaction flask, 33.46g(87.30mmol) of N^ϵ-(4-methoxybenzoyl)-9-(t-butoxycarbonylmethyl) adenine and 800ml of dichloromethane were placed, mixed at room temperature, and added 180ml of TFA. The reactants were mixed overnight at the same temperature with constant stirring. After dichloromethane and TFA were removed under reduced vacuum, the resulting

crude compound was solublized in 450ml of 1N NaOH. The solution was washed with dichloromethane and acidified with 6N HCl to obtain 23.54g(71.9mmol) of N^6-(4-methoxybenzoyl)-9-(carbonylmethyl) adenine as as white solid.   In a 500ml

5   reaction flask, 21.99g(67.20mmol) of N^6-(4-methoxybenzoyl)-9-(carbonylmethyl) adenine, 20.72g(62.7mmol) of benzyl N-[2-(t-butoxyl)aminoethyl]   glycinate   and   200ml   of dichloromethane were added sequentially, and then added 14.17g(73.92mmol)   of   1-(3-dimethylaminopropyl)-3-

10 ethylcarbodiamide   at   once.   The   reaction   mixture   was incubated   overnight   at   room   temperature   with   constant stirring,   extracted   with   dichloromethane   and   water,   and washed with brine.   The organic phase layer was dried with magnesium sulfate, and concentrated under reduced vacuum to

15 obtain crude compound, which was subsequently purified through flash column chromatography by using a mobile phase of 5% methanol/dichloromethane to give 28.24g(45.70mol) of benzyl   N-[2-(t-butoxylcarbonyl)aminoethyl]-N-[(N$^\epsilon$-(4-methoxybenzoyl)-adenine-9-yl) acetyl]glycinate as a white

20 powder. 400ml of methanol was added to 28.24g(45.70mol) of the said compound, and then added 1.0g of palladium on activated   carbon(Pd/C)   with   stirring.   Under   hydrogen environment created by hydrogen balloon, the reaction flask was stirred for 3 hours.   The catalyst was removed by

25 filtering though celite, and evaporated the solvent under reduced vacuum.   The resultant material was solubilized in 200ml of 1N NaOH, washed with dichloromethane, acidified with 6N HCl and recrystallized in dichloromethane and hexane, respectively, to obtain 17.07g(32.40mmol) of N-[2-

30 (t-butoxycarbonyl)aminoethyl]-N-[(N^2-(4-methoxybenzoyl)-adenine-9-yl)   acetyl]glycine(white   powder)   as   a   titled product.

^{1}H NMR(DMSO-D$_6$) δ   11.0(s, 1H), 8.67(s, 1H), 8.30(S, 1H),

35   8.05(d, 2H), 7.08(d, 2H), 5.16(2xs, 2H),
                                 3.98(2xs, 2H), 3.84(s, 3H), 3.04(m, 1H),
                                 1.38(s, 9H)

Preparative Example 3-3: Guanine monomer

After 3.02g(20mmol) of guanine was added into 60ml
of DMF, 6.2ml(44mmol) of triethylamine and 4.6ml(44mmol) of
isobutylyl chloride were added, and the reaction was
carried out overnight at 120°C with constant stirring.
Then, the reaction temperature was lowered to room
temperature and the reaction was stopped by addition of
20ml of methanol. The reaction solution was concentrated
under reduced vacuum, and precipitated by adding hot
isopropanol. The precipitate was filtered and separated to
obtain 1.84g(8.32mmol) of N^2-(isobutanoyl) guanine as a
white powder. To 1.83g(8.27mmol) of N^2-(isobutanoyl)
guanine, was added 50ml of DMF and 0.40g(10mmol) of 60%
NaH. The resulting suspension was stirred for 1 hour at
room temperature, added 1.35ml(9.14mmol) of t-
butylbromoacetate and mixed with constant stirring for 2
more hours at the same temperature. The resulting solution
was then treated with CO_2/methanol, concentrated under
reduced vacuum to otain the crude compound. The crude
compound was extracted with ethylacetate, and the resulting
organic phase layer was concentrated under reduced vacuum
to give a mixture of N^2-(isobutanoyl)-9-(t-
butoxycarbonylmethyl) guanine and N^2-(isobutanoyl)-7-(t-
butoxycarbonylmethyl) guanine as a white powder. To the
mixture, were added 50ml of dichloromethane and 19ml of TFA
with stirring. The final reaction mixture was incubated
for 2 days with constant stirring, and concentrated under
reduced vacuum, to obtain a mixture of 1.5g(5.64mmol) of
N^2-(isobutanoyl)-9-(carboxylmethyl) guanine and N^2-
(isobutanoyl)-7-(carboxylmethyl) guanine. The said mixture
was treated with ethylacetate to give a white precipitate,
and filtered and dried under reduced vacuum, to give
0.72g(2.57mmol) of N^2-(isobutanoyl)-9-(carboxylmethyl)
guanine. 690mg(2.47mmol) of the said compound,
922mg(2.99mmol) of benzyl N-[2-(t-butoxyl)aminoethyl]

glycinate and 577mg(3.01mmol) of EDC were dissolved in 20ml of DMF, and the reaction mixture was stirred for 3 hours at room temperature with constant stirring. After DMF was removed under reduced vacuum, the resultant compound was extracted with ethylacetate, and the organic phase layer was concentrated under reduced vacuum. The crude compound was solubilized in 10ml dichloromethane, and recrystallized in hexane to obtain white precipitate. The precipitate was separated and further purified through flash column chromatography by using a mobile phase of 10% methanol/dichloromethane to give 1.24g(2.18mmol) of benzyl N-[2-(t-butoxylcarbonyl)aminoethyl]-N-[(N^2-(isobutanoyl)-guanine-9-yl) acetyl]glycinate. 25ml of methanol was added to 1.22g(2.14mmol) of said compound, and then mixed with 0.25g of 10% Pd/C with stirring. Under hydrogen environment created by hydrogen balloon, the reaction flask was stirred for 2 hours and 45 minutes. The catalyst was removed by filtering though celite, and the filtrate was concentrated under reduced vacuum. The crude compound was dissolved in ethylacetate and stirred overnight to obtain a white precipitate. The precipitate was filtered and dried to obtain 0.92g(1.91mmol) of N-[2-(t-butoxylcarbonyl)aminoethyl]-N-[(N^2-(isobutanoyl)-guanine-9-yl) acetyl]glycine in a white powder form.

^{1}H NMR(DMSO-D$_6$) δ   7.82(2xs, 1H), 7.03~6.81(m, 2H),
                         4.94(2xs, 2H), 3.99(2xs, 2H),
                         3.04(d, 1H), 3.19(2xt, 2H), 2.89(t, 2H),
                         2.78(m, 1H) 1.19(d, 6H)

Preparative Example 3-4: Cytosine monomer

22.02g(0.20mol) of cytosine was dissolved in pyridine, added 37.53g(0.22mol) of p-anisole chloride slowly over 45 minutes. Then, the reaction temperature was slowly elevated to 120°C, and reacted overnight at the same temperature with constant stirring. The crude product was

obtained by concentrating the resultant under reduced
vacuum. The crude product was then solubilized in methanol,
heated and cooled to obtain the precipitate. The
precipitate was filtered and dried to obtain
25.78g(0.11mol) of N^4-(4-methoxybenzoyl) cytosine. To
25.75g(0.11mol) of N^4-(4-methoxybenzoyl) cytosine in a 1
liter reaction flask was added 500ml of DMF, then added
5.04g(0.13mol) of 60% NaH and 18.61ml(0.13mol) of t-
buthylbromoacetate sequentially. After the reaction
mixture was stirred overnight at room temperature, DMF was
removed under reduced vacuum. The crude product thus
obtained was recrystallized in methanol to obtain
22.52g(62.60mmol) of 1-(t-butoxycarbonylmethyl)-N^4-(4-
methoxybenzoyl) cytosine as a white powder.
22.52g(62.60mmol) of the said compound and 600ml of
dichloromethane were put into a 2 liter reaction flask,
mixed at room temperature, and then added 130ml of TFA.
The reaction mixture was stirred overnight at room
temperature. After dichloromethane and TFA were removed
under reduced vacuum, the resulting crude compound was
solubilized in 450ml of 1N NaOH, acidified with 6N HCl to
obtain a white precipitate. The precipitate was filtered
and dried to obtain 18.82g(62.10mmol) of 1-
(carboxylmethyl)-N^4-(4-methoxybenzoyl) cytosine as a white
powder. 18.82g(62.10mmol) of the said compound was mixed
with 12.05g(68.3mmmol) of benzyl N-[2-(t-
butoxylcarbonyl)aminoethyl] glycinate in a 500 ml reaction
flask, and then sequentially with 120ml of DMF and with
13.09g(68.30mmol) of EDC. The reaction mixture was
stirred overnight at room temperature, and then DMF solvent
was removed under reduced vacuum. The resulting crude
product was extracted with dichloromethane, concentrated
under reduced vacuum, and recrystallized in dichloromethane
and hexane, respectively. 26.90g(45.30mmol) of benzyl N-
[2-(t-butoxycarbonyl)aminoethyl]-N-[(N^4-(4-methoxybenzoyl)-
cytosine-1-yl) acetyl]glycinate was obtained as a white
precipitate. 26.90g(45.30mmol) of the said compound was

dissolved in 850ml of methanol, and then added 1.0g of Pd/C
with stirring. The reaction solution was stirred for 4
hours under hydrogen environment created by hydrogen
balloon. The catalyst was removed by filtering though
5 celite, and the filtrate was concentrated under reduced
vacuum. The resulting material was then solubilized in
200ml of 1N NaOH, washed with dichloromethane, acidified
with 6N HCl, recrystallized in dichloromethane and hexane,
respectively, to obtain 18.83g(37.40mmol) of N-[2-(t-
10 butoxycarbonyl)aminoethyl]-N-[(N^4-(4-methoxybenzoyl)-
cytosine-1-yl) acetyl]glycine as a white powder.

^{1}H NMR(DMSO-D$_6$) δ   8.03(d, 2H), 7.95(2xs, 1H),
                          7.30(2xs, 1H), 7.04(d, 2H),
15   4.84~4.66(2xs, 2H), 4.20~3.98(2xs, 2H),
                          3.82(s, 3H), 3.22~3.03(t, 4H),
                          1.37(s, 9H)

Preparative Example 3-5: Thymine monomer

20   After 50.45g(0.40mol) of thymine, 55.28g(0.40mol)
potassium carbonate and 1.20ml of DMF were mixed in a 2
liter round flask, 44.40ml(0.40mol) of ethylbromoacetate
was added at once to the resulting white suspension, and
25 stirred for 48 hours at room temperature. The
unsolubilized solid was removed by filtering, and DMF in
the filtrate was eliminated under reduced vacuum. After
the precipitate was formed by lowering the reaction
temperature to 0°C, this precipitate was mixed with 400ml
30 of water and 11ml of 6N HCl for 30 minutes with stirring.
The white precipitate of 1-(ethoxycarbonylmethyl)thymine
was obtained by filtering. The said compound was then
mixed with 400ml of water and 200ml of 2N NaOH in a 1 liter
reaction flask, and refluxed for 10 minutes with heating.
35 When the white suspension solution became clear during
heating, the reaction solution was cooled down to 0°C, and
added 90ml of 6N HCl to give a white precipitate. The said

precipitate was filtered, washed with water, and dried in vacuum oven to obtain 47.56g(0.26mol) of 1-(carboxymethyl) thymine as a white powder. 10.94g(54.40mmol) of the said compound and 15.27g(49.50mmol) of benzyl N-[2-(t-butoxycarbonyl)aminoethyl] glycinate were then dissolved in 250 ml of DMF. And then, 11.39g(59.40mmol) of EDC was added and stirred overnight at room temperature. After DMF in the reaction solution was removed under reduced vacuum, 500ml of water was added to the remaining solution and extracted with dichloromethane. The resulting organic phase was dried with magnesium sulfate, concentrated under reduced vacuum to obtain crude compound. The crude compound was purified through flash column chromatography by using a mobile phase of 10% methanol/dichloromethane to give 21.76g(45.90mmol) of benzyl N-[2-(t-butoxycarbonyl)aminoethyl]-N-((thymine-1-yl) acetyl]glycinate as a white powder. 21.76g(45.90mmol) of the said compound was dissolved in 400ml of methanol, and mixed with 10% of 5.0g Pd/C. Then, the reaction mixture was further stirred under hydrogen environment created by hydrogen balloon for 4 hours and 30 minutes. The catalyst was removed by filtering though celite, and the filtrate was added with 50ml of 1N NaOH and 100ml of water, followed by washing with dichloromethane and acidification with 6N HCl. The resulting mixture was extracted with ethylacetate. The organic phase was collected, dried with magnesium sulfate, and concentrated under a reduced vacuum, then further dried in a vacuum oven to obtain 17.05g(43.60mmol) of N-[2-(t-butoxycarbonyl)aminoethyl]-N-(thymine-1-yl) acetyl]glycine in a white powder form.

^{1}H NMR(DMSO-D$_6$) δ   11.30(d, 1H), 7.30(2xs, 1H),
                          4.64~4.46(2xs, 2H), 4.19~4.01(2xs, 2H),
                          3.34(s, 2H), 3.38~3.30(t, 2H),
                          3.17~2.89(t, 2H), 1.91(s, 3H),
                          1.37(s, 9H)

The process for preparing arrays of peptide nucleic acid with various nucleotide sequences using polymeric photoacid generator will be illustrated in more detail.

5 <u>Step 1</u>: Attachment of linker on the solid matrix

The surface of a solid matrix is derivatized with aminoalkyloxysilane in alcohol, and the linker with acid-labile protecting group is attached on the solid matrix.

10 First, to fix amine group on the surface of solid matrix, e.g., plate made of material containing the organic solvent resistance such as silicon, surface-derivatized glass, polypropylene, or activated acrylamide, preferably surface-derivatized glass, more preferably glass plate for

15 microscope, the solid matrix is immersed in 0.05 to 0.15%(v/v), most preferably 0.1%(v/v) of aminoalkyloxysilane solution, proferably aminopropyltriethoxysilane in alcohol (most preferably, in ethanol), mixed for 3 to 7 minutes, more perferably 5

20 minutes, at room temperature, washed with alcohol, most preferably ethanol, and dried in the vacuum oven at 100 to 140°C, more preferably at 110 to 130°C, most preferably 120°C, for 10 to 30 minutes, preferably 15 to 25 minutes, most preferably 20 minutes. Then, it is placed under argon

25 gas for 10 to 14 hours, most preferably for 12 hours, immersed in DMF, and washed thoroughly with dichloromethane.

Secondly, to attach the linker with acid-labile protecting group to the amine group fixed on the the solid matrix, 0.02 to 0.2mM, most preferably 0.1mM of

30 aminoalkylcarboxylic acid solution, most preferably 6-N-t-butoxycarbonylamino carproic acid, in N-methylpyrrolidone containing 0.05 to 0.5mM, more preferably 0.2mM of diisopropylethylamine is prepared, and mixed with 0.05-0.5mM, most preferably 0.12mM of HATU (o-(7-

35 azabenzotriazol-1-yl)-1,1,3,3-tetramethyluroniumhexafluoro -phosphate) in NMP. After mixing the above two solutions, it is activated for 1 to 5 minutes, most preferably for 2

minutes, the solid matrix thus prepared is immersed and incubated for 30 minutes to 4 hours, preferably 1 to 3 hours, most preferably 2 hours, at 40 to 90°C, most preferably 60°C, with agitation. The unreacted amine group
5 on the solid matrix is capped with acetyl group by incubating in 1:2 to 1:4 (v/v), most preferably 1:3(v/v) of acetic anhydride/pyridine solution for 0.5 to 2 hours, most preferably 1 hour, with agitation.

10 Step 2: Coating with polymeric PAG

The solid matrix thus prepared is coated with polymeric PAG: the solid matrix to which the linker or monomer is attached is spin-coated with 5 to 50%(w/v)
15 polymeric PAG in organic solvent, most preferably NMP, at 1,000 to 5,000rpm, most preferably 3,000rpm.

Step 3: Removal of acid-labile protection group

20 The solid matrix thus coated is exposed to light to generate acid, to eliminate acid-labile protection group: the coated solid matrix is soft-baked for 1 minute at 50 to 100°C, most preferably for 30 seconds to 2 minutes at 80°C, and exposed to white light of short wavelength for 6
25 seconds to 2 minutes, most preferably for 1 minute, using photomask, baked again for 3 minute at 50 to 100°C, most preferably for 30 seconds to 5 minutes at 80°C to generate acid, finally to remove protecting group at the exposed location selectively.

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Step 4: Removal of residual polymeric PAG

The solid matrix thus prepared is washed with alkaline solution or organic solvent, and residual
35 polymeric PAG is removed: after completing the Step 3, residual polymeric PAG is washed and removed with diluted trialkylammoniumhydroxide, NaOH solution, KOH solution or

organic solvent.

<u>Step 5</u>: Reaction with peptide nucleic acid monomer

A monomeric peptide nucleic acid with acid-labile protecting group is attached to the solid matrix thus prepared: the selectively exposed amine group on the solid matrix is reacted with the peptide nucleic acid monomer prepared in Preparative Example 3. An NMP solution of 10-20μM of peptide nucleic acid monomer, most preferably 15μM, with diisopropylethylamine at a ratio of 1:1 to 1:5(w/w) to the solubilized nucleic acid monomer, and another NMP solution containing HATU at a ratio of 1:1 to 1:2(w/w) to the solubilized nucleic acid monomer in the said mixed solution are prepared. The two solutions are mixed, activated for 1 to 5 minutes, and reacted with the solid matrix thus prepared at 50 to 100°C, most preferably at 60°C for 1 to 3 hours, most preferably for 2 hours, with agitation. The unreacted amine group is capped with acetyl group by incubating in 1:2 to 1:4(v/v), most preferably 1:3(v/v), of acetic anhydride/pyridine solution for 0.5 to 2 hours, most preferably 1 hour, with agitation.

Then, arrays of peptide nucleic acid probes immobilized on the solid matrix are finally prepared by repeating Steps 2 to 5.

The free reactive group on the solid matrix, i.e. the amine group of peptide nucleic acid, is tagged with the fluorescent tag by incubating with 0.5 to 2mM, most preferably 1mM, of fluorescent isothiocyanate in DMF for 30 minutes to 2 hours, most preferably 1 hour, at room temperature, followed by washing with DMF and ethanol sequentially. It is kept in the dark until analyzed by confocal fluorescent laser.

The process for preparing arrays of peptide nucleic acid with various nucleotide sequences by employing

polymeric PAG has advantages over prior art as followings:

First, the invented process is desirable in a sense that spin-coating of polymeric PAG dissolved in a proper solvent can be carried out directly, while prior art PPA process employs monomeric PAG mixed with polymeric matrix.

Secondly, the acid generated in PPA process acts seperately from the polymeric matrix and the actual amount of acid for deprotecting become less, while PAG itself, in the invented process employing polymeric PAG, contacts with the group to be deprotected and more amount of the acid generated takes part in deprotecting reaction.

Thirdly, PAG can be attached directly to polymeric backbone, which makes PAG become stable to the heat or chemicals generated during the process, assuring that a good quality pattern of a minimum thickness can be obtained without domain formation during coating, which in turn facilitates generation and diffusion of acid by making the exposed light reach the bottom easily.

Fourthly, the process is economical since polymeric PAG can be washed with diluted alkaline solution after patterning.

The present invention is further illustrated by the following examples, which should not be taken to limit the scope of the invention.

Example 1: preparation of peptide nucleic acid probe

Example 1-1: Attachment of linker on the glass plate

A glass plate for microscope was immersed in cleansing solution (containing 1L of 95% ethanol, water 12ml, NaOH 120g), rinsed several times with water and air-dried. Then, the glass plate was rinsed with 95% ethanol, immersed in 0.1%(v/v) aminopropyltriethoxysilane in ethanol for 5 minutes at room temperature with agitation, rinsed again with 95% ethanol for three times, and dried in vacuum.

oven maintained at 120°C for 20 minutes. And then, the glass plate was placed under argon gas for 12 hours, immersed in DMF, rinsed with dichloromethane, and amine group was fixed on the solid matrix of glass plate for microscope.

Subsequently, 0.5ml of NMP solution containing 0.1mM 6-N-t-butoxycarbonyamino caproic acid and 0.2mM diisopropylethylamine was mixed with NMP solution containing 0.12mM HATU for 2 minutes. The linker was attached on the surface-derivatized glass plate by immersing the solid matrix prepared above into the said mixed solution for 2 hours at 60°C with agitation. Then, unreacted amine group was capped with acetyl group by incubating in acetic anhydride/pyridine (1:3, v/v) solution for 1 hour with agitation.

Example 1-2: Attachment of peptide nucleic acid

Polymeric PAG obtained in Preparative Example 2 was dissolved in NMP to reach a final concentration of 30%(w/v), and spin-coated at 3,000rpm on the glass plate with linker containing acid-labile protecting group prepared in Example 1-1. The coated glass plate was soft-baked for 1 minute at 80°C, exposed to white light with short wavelength using photomask, post baked for 3 minutes at 80°C, and selectively removed the protecting group at the exposed locations by generating acid.

Then, the detached protecting group and the residual polymeric PAG on the glass plate were washed out with 1% trialkylammoniumhydroxide. A solution with 0.3g of diisopropylethylamine dissolved in NMP solution containing 0.3g of peptide nucleic acid monomer prepared in Preparative Example 3 and another solution with 0.4g HATU dissolved in the NMP solution were mixed, and activated for 2 minutes. The glass plate with having its protecting group selectively removed was immersed in the activated solution for 2 hours with agitation, and the peptide

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nucleic acid monomer was linked to the deprotected amine group on the glass plate. The unreacted amine group was capped with acetyl group by incubating in acetic anhydride/pyridine (1:3, v/v) solution for 1 hour with
5 agitation.

Then, repeating the process of PAG polymeric coating, light exposure using photomask, removal of protection group, reaction of petide nucleic acid mononer having protected amine group and capping, the peptide nucleic acid on the
10 glass plate was elongated to obtain desired length.

The free reactive group on the glass plate was tagged with fluorescence by incubating with 1mM of fluorescent isothiocyanate in DMF for 1 hour at room
15 temperature, washed with DMF and ethanol sequentially, dried and kept in the dark. The microscopic image in the 10μ resolution can be obtained by analyzing the glass plate with spectrofluorimeter.

20 As clearly described and demonstrated as above, the present invention provides a process for preparing arrays of peptide nucleic acid probe with various nucleotide sequences immobilized on a solid matrix that can be used for DNA chip fabrication. In accordance with the present
25 invention, neutral peptide nucleic acid probes, as the promising substitute for conventional negatively charged oligonucleotide probes, can be prepared by employing polymeric photoacid generator in a simple and efficient manner, while overcoming the problems confronted in the
30 prior art DNA chip fabrication using PR system and PPA system.

It will be apparent to those skilled in the art that certain changes and modifications can be made to this
35 invention without departing from the spirit or scope of the invention as it is set forth herein.

<u>WHAT IS CLAIMED IS</u>:

1. A process for preparing arrays of peptide nucleic acid probes immobilized on a solid matrix by employing polymeric photoacid generator which comprises the steps of:

(i) derivatizing the surface of a solid matrix with aminoalkyloxysilane in alcohol and attaching a linker with acid-labile protecting group on the solid matrix;

(ii) coating the solid matrix with polymeric photoacid generator(PAG);

(iii) exposing the solid matrix thus coated to light to generate acid for eliminating acid-labile protecting group;

(iv) washing the solid matrix with alkaline solution or organic solvent and removing residual polymeric photoacid generator; and,

(v) attaching a monomeric peptide nucleic acid with acid-labile protecting group to the solid matrix, and repeating the previous Steps of (ii) to (v).

2. The process for preparing arrays of peptide nucleic acid probes immobilized on a solid matrix of claim 1, wherein the solid matrix is a plate made of material with the organic solvent resistance such as silicone, surface-derivatized glass, polypropylene or activated acrylamide.

3. The process for preparing arrays of peptide nucleic acid probes immobilized on a solid matrix of claim 1, wherein the surface of the solid matrix is derivatized by immersing in 0.05 to 0.15%(v/v) of aminoalkyloxysilane in alcohol; mixing for 3 to 7 minutes at room temperature; washing with alcohol and drying at 100 to 140°C for 10 to 30 minutes; placing under argon gas for 10 to 14 hours; and, washing with dimethylformamide and dichloromethane, to obtain amine group-fixed solid matrix.

4. The process for preparing arrays of peptide nucleic

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acid probes immobilized on a solid matrix of claim 1,
wherein the linker with acid-labile protecting group is
aminoalkylcarboxylic acid.

5   5.   The process for preparing arrays of peptide
nucleic acid probes immobilized on a solid matrix of claim
1, wherein the attaching of linker to amine group on the
solid matrix is carried out by mixing a solution of 0.02 to
0.2mM aminoalkylcarboxylic acid in NMP(N-methylpyrrolidone)
10   and 0.05 to 0.5mM diisopropylethylamine with a solution of
0.05 to 0.5mM of HATU (o-(7-azabenzotriazol-1-yl)-1,1,3,3-
tetramethyluroniumhexafluorophosphate) in NMP; activating
for 1 to 5 minutes, immersing a solid matrix in the
activated solution and incubating for 30 minutes to 4 hours
15   at 40 to 90°C with agitation; and, capping unreacted amine
group on the matrix with acetyl group by incubating thus
incubated   matrix   in   1:2   to   1:4(v/v)   of   acetic
anhydride/pyridine solution for 0.5 to 2 hours with
agitation.
20

6. The process for preparing preparing arrays of
peptide nucleic acid probes immobilized on a solid matrix
of claim 1, wherein the polymeric photoacid generator is a
polymer with molecular weight of 500 to 1,000,000, which
25   has sulfonium salt on backbone or side chain, and has also
organic photoacid generating group on side chain to
generate acid by the exposure of light.

7. The process for preparing preparing arrays of
30   ,peptide nucleic acid probes immobilized on a solid matrix
of claim 1, wherein the coating of solid matrix with
polymeric PAG is carried out by spin-coating the solid
matrix to which a linker or monomer is attached, with 5 to
50%(w/v) polymeric PAG in organic solvent at a speed of
35   1,000 to 5,000 rpm.

8. The process for preparing arrays of peptide

nucleic acid probes immobilized on a solid matrix of claim 1, wherein the elimination of acid-labile protecting group is carried out by soft-baking a coated solid matrix at 50 to 100°C for 30 second to 2 minutes; exposing to white
5   light of short wavelength for 30 seconds to 2 minutes using photomask; and, baking again at 50 to 100°C for 30 seconds to 5 minutes to generate acid.

9. The process for preparing arrays of peptide
10   nucleic acid probes immobilized on a solid matrix of claim 1, wherein the monomer is a compound, whose amine group located in backbone is protected by t-butyloxycarbonyl group, and amine group located in base by p-methoxybenzoyl or isobutanoyl group.

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10. The process for preparing arrays of peptide nucleic acid probes immobilized on a solid matrix of claim 1, wherein the alkaline solution in Step (iv) is diluted trialkylammoniumhydroxide, NaOH solution or KOH solution.

20

11. The process for preparing arrays of peptide nucleic acid probes immobilized on a solid matrix of claim 1, wherein the attaching of a peptide nucleic acid monomer with acid-labile protecting group to a solid matrix in Step
25   (v) is carried out by mixing an NMP solution containing 10-20μM of peptide nucleic acid monomer and diisopropylethylamine at a ratio of 1:1 to 1:5(w/w) and an NMP solution containing HATU at a ratio of 1:1 to 1:2(w/w) based on the nucleic acid monomer; activating for 1 to 5
30 . minutes; immersing the acid-labile.protecting group free solid matrix in the activated solution; incubating at 50 to 100°C for 1 to 3 hours with agitation; and, capping unreacted amine group on the matrix with acetyl group by incubating thus incubated matrix in 1:2 to 1:4(v/v) of
35   acetic anhydride/pyridine solution for 0.5 to 2 hours with agitation.

12. Arrays of peptide nucleic acid probes on a solid matrix prepared by the process of claim 1.

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC7 C12Q 1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimun documentation searched (classification system followed by classification symbols)

IPC C12Q 1/68, GO1N 33/543, GO1N 33/552, GO1N 33/544

Documentation searched other than minimun documentation to the extent that such documents are included in the fileds searched

Korean Patents and Applications for inventions since 1975

Electronic data base consulted during the intertnational search (name of data base and, where practicable, search trerms used)
IBM, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 6,060,242 (Lorne Park Research, Inc.) 09 May 2000 see the whole document	1-12
A	US 5,843,655 (Affymetrix, Inc.) 01 December 1998 see the whole document	1-12
A	US 5,143,854 (Affymax Tech.) 01 September 1992 see the whole document	1-12

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevence "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevence; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevence; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art "&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 SEPTEMBER 2000 (20.09.2000)	25 SEPTEMBER 2000 (25.09.2000)

| Name and mailing address of the ISA/KR
 Korean Industrial Property Office
 Government Complex-Taejon, Dunsan-dong, So-ku, Taejon
 Metropolitan City 302-701, Republic of Korea
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 Telephone No. 82-42-481-5593 |

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